A Simplified Kinetic Model for Enzymatic Hydrolysis of Bovine Serum Albumin

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On the basis of the mechanism of protein enzymatic hydrolysis, the hydrolysates were combined into several lumps according to their molecular mass. The reaction of lumps was described with a simplified kinetic equation. Then the kinetic model for bovine serum albumin tryptic hydrolysis reaction was established. The rate constant of each reaction was estimated by a nonlinear regression rule in two steps. The ability of prediction and extrapolation of the simplified kinetic model and the good quality of the obtained rate coefficients was tested by comparing the computed values with the experimental values.

Key words:
Bovine serum albumin, hydrolysis, enzyme, kinetic model

Introduction

Protein hydrolysates have been applied as functional and nutritional ingredients in food, pharmaceutical and cosmetic industries during the last two decades. Many bioactive peptides have been isolated from enzymatic digests of various food and blood proteins. For example, insulin-stimulating peptide,[1] which can enhance the action of insulin in vitro on fatty acid synthesis, has been obtained from a tryptic digest of bovine serum albumin (BSA). Bovine albutensin A[2] is an ileum-contracting peptide isolated from the tryptic hydrolysates of BSA, which exhibits ileal contraction in the longitudinal muscle strips of guinea pig ileum. Neurotensin,[3] got from pepsin-treated BSA, is a tridecapeptide in the regulation of central dopaminergic systems and gastrointenstinal function.

The kinetic model of protein hydrolysis reaction is one of the important aspects in the related study because it deals with the evaluation of the enzyme, the mechanism of enzymatic hydrolysis, etc. Disappointedly, previous studies focused on the product development, and the kinetic model rested on the Michaelis-Menten equation which only can describes the one-substrate enzymatic reaction. Protein enzymatic hydrolysis is a kind of complicated reaction system that contains multi-component and multi-reaction. In the reaction, a component produced from a hydrolysis reaction, is perhaps the substrate for another reaction at the same time. A substrate can probably generate several kinds of product. Parallel reaction exists with consecutive reaction simultaneously, and the reactions are coupled and restricted with each other. Due to the complexity of protein hydrolysis reaction, successful kinetic model must rely on the lumping technique that systematically reduces the number of variables and parameters. The lumping technique has been widely used in petrochemical industries,[4–6] and has been attempted to be used in biochemical reaction system.[7] It combines groups of components whose kinetic behavior are similar into several lumps to simplify the complicated system. In this work, the hydrolysates were combined into several lumps according to their molecular mass to simplify the kinetic model for BSA enzymatic hydrolysis reaction. And the lumping method can be applied to the investigation of reaction kinetics of similar complicated reaction systems such as polysaccharides, lipids or nucleic acids.

Mechanism of protein enzymatic hydrolysis

The mechanism of protein enzymatic hydrolysis is the basis of kinetic model. Trypsin is a kind of high specificity protease that only attacks the long alkaline amino acid residues such as argenine and lysine and cuts off the protein chain at the carbonyl end. As a result, the cleavage of peptide obeys some rules. In the hydrolysis process, molecular mass of protein and peptide is an important factor that influences the dynamic performance of reactants. Generally speaking, the volume of substrates is large when its molecular mass is large. It is difficult to band with the enzyme molecule because of

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the volume barrier. But the number of the reactable amino acid residue in large molecule substrate is larger than that of the small molecule substrate, which will contribute to the rise of reaction rate. Therefore, the reaction rate is determined by two aspects. And it can be concluded that the reactants whose molecular mass are similar to each other have similar dynamic performance.

The mechanism of protein enzymatic hydrolysis can be described by the following steps. First, free enzyme combines with substrate to form active middle complex. Then the peptide chain cracks and the complex decomposes to two products. At the same time the enzyme is set free. This is the rate deterministic step as shown in Eq. 1. Product inhibition and substrate inhibition exist in the hydrolysis reaction, which can be expressed by Eq. 2 and 3.

$$E + S \underset{}{\overset{K_m}{\rightleftarrows}} E + P \quad (1)$$

$$ES + S \overset{K_s}{\rightleftarrows} ES_2 \quad (2)$$

$$E + P \overset{K_p}{\rightleftarrows} EP \quad (3)$$

The inactivation of enzyme fits well with the first-order model:[8] $\frac{d\gamma_E}{dt} = -k_d \gamma_E$.

Taking into account product inhibition, substrate inhibition and enzyme deactivation, the performance of each lump in the system can be described by following equation:

$$\frac{d\gamma_S}{dt} = k\gamma_{ES} = \frac{k_{cat} + \gamma_S \gamma_{E_0} \exp(-k_d t)}{K_m\left(1 + \frac{\gamma_P}{K_p}\right) + \gamma_S\left(1 + \frac{\gamma_S}{K_S}\right)} \quad (4)$$

Establishment of lumping kinetic model

Basic hypothesis

Before the establishment of the model, some hypotheses have been put forward as follows:

A) Enzymatic hydrolysis reaction is affected by the space structure of substrate, the specificity of enzyme and other factors. So the cleavage position of peptide is not random, and the molecular mass contribution is regular.

B) In the same reaction system, dynamic performances of substrates are similar if their molecular mass are alike.

C) Product inhibition, substrate inhibition and enzyme deactivation exist in the hydrolysis process. And the kinetic behavior of each lump can be described by the modified Michaelis-Menten equation, i.e., Eq. (4.)

D) There is no interaction for the products, which means that enzymatic synthesis does not take place.

E) The lumps which molecular mass are large can yield the smaller lumps. The lump which molecular mass is the smallest does not hydrolyze.

F) The reaction is carried out in homogeneous phase, and the influence of diffuse resistance does not exist.

Lumping method

Based on the data obtained from high performance size-exclusive chromatogram (HPSEC), the products of tryptic hydrolysis of BSA can be combined into several lumps. Figure 1 is the chromatograph of BSA tryptic hydrolysates.

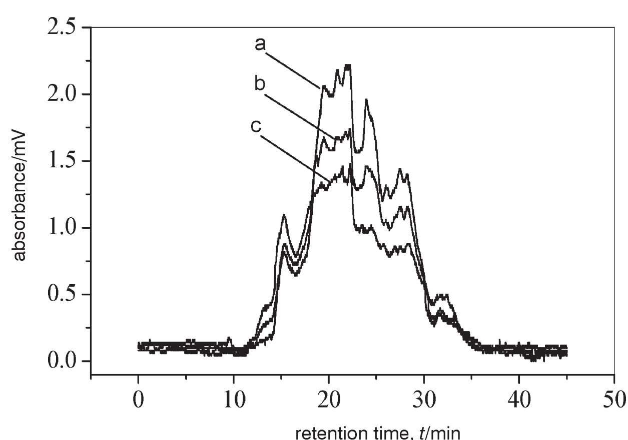


Fig. 1 – *HPSEC analysis of bovine serum albumin tryptic hydrolysates after 2.5 min (curve a), 20 min (curve b) and 60 min (curve c)*

There are four districts in the chromatogram corresponding to molecular mass range: 127,000~24,000, 24,000~6,300, 6,300~3,000 and 3,000~300. The areas of these peaks varied with time. The area of the first district decreased while that of other districts increased. This illuminates that hydrolysis rates of the products in a district are close to each other. Thus, the products of BSA tryptic hydrolysis were combined into four lumps according to the analysis results of HPSEC.

Establishment of reaction network

After all components were grouped into several lumps, the network for BSA hydrolysis can be built up based on the mechanism and the hypothesis, and is shown in Figure 2. The kinetic model contains four lumps and six kinetic parameters. The lump 'A' represents the components whose molecular mass are the biggest, and the lump 'D' represents the smallest.

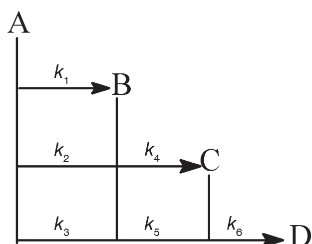


Fig. 2 – *Four lumps network for BSA tryptic hydrolysis*

Due to the increase of product and the decrease of substrate in the reaction, the influence of product inhibition to the reaction was rising while the influence of substrate inhibition was declining. As a result, the value of the denominator of Eq. 4 does not change significantly over the reaction time. Table 1 shows the equilibrium constants K_m, K_p and K_s of each lump at 40 °C which were measured by experiments. The reaction rate of lump D is zero because it does not hydrolyze any more. Table 2 presents the values of the denominator of Eq. (4) for each lump at different times at 40 °C. If the change of the values is ignored, Eq. (4) can be simplified as Eq. (5). Compared with the intrinsic Eq. (4), the parameters of Eq. (5) were reduced. Therefore, a lot of experimental work to determine the rate constants of K_m, K_p and K_s can be spared. But the Eq. (5) can not describe the mechanism of reaction.

Table 1 – *The equilibrium constants K_m, K_p and K_s of each lump*[a]

Lump	K_m	K_p	K_s
A	0.422	0	13.420
B	0.828	0.493	11.257
C	2.438	0.319	9.962
D	0	0.176	0

[a]The unit of K_m, K_p and K_s is mg m L^{-1}

Table 2 – *The value of the denominator of Eq. (4) of each lump*

Lump	5 min	20 min	40 min	60 min	90 min
A	5.382	5.292	5.142	5.212	5.288
B	4.518	5.003	5.357	5.619	5.817
C	6.619	7.121	7.486	7.728	8.054

$$\frac{d\gamma_S}{dt} = k\gamma_{ES} = k'\gamma_S\gamma_{E_0}\exp(-k_d t) \qquad (5)$$

where $k' = k/(K_m(1+\gamma_p/K_p)+\gamma_s(1+\gamma_s/K_s))$. The values of k_d at different temperatures have been measured by experiments. The values are as follows: 0.00531 min^{-1}, 0.00815 min^{-1}, 0.0120 min^{-1}, 0.0183 min^{-1} and 0.0288 min^{-1} at 30 °C, 35 °C, 40 °C, 45 °C and 50 °C, respectively.

Then the differential equation set, which describe the reaction network of BSA hydrolysis, is shown as follows.

$$\frac{d\gamma_A}{dt} = -\gamma_{E_0}\exp(-k_d t)(k_1+k_2+k_3)\gamma_A$$

$$\frac{d\gamma_B}{dt} = \gamma_{E_0}\exp(-k_d t)(k_1\gamma_A-(k_4+k_5)\gamma_B)$$

$$\frac{d\gamma_C}{dt} = \gamma_{E_0}\exp(-k_d t)(k_3\gamma_A+k_4\gamma_B-k_6\gamma_C)$$

$$\frac{d\gamma_D}{dt} = \gamma_{E_0}\exp(-k_d t)(k_3\gamma_A+k_5\gamma_B+k_6\gamma_C)$$

Parameter estimation of kinetic model

Experimental procedure

Experimental conditions for quantity estimation: BSA (4.0 mg mL^{-1}) was hydrolyzed with trypsin ($\zeta_{enz./sub.}$ = 1/100) in a batch stirred tank reactor. The reaction was carried out at 30 °C, 40 °C and 50 °C, respectively. The pH was kept at 8.0 by adding 0.1 mol L^{-1} NaOH solution with the pH-stat method. Samples were taken at different times, and hydrolysis reaction was stopped by heating in boiling water for ten minutes. Then the molecular mass distribution of the samples was analyzed by HPSEC. The mass concentration of each lump was determined by HPSEC at the same time. The results showed that the error is less than 5 %. In addition, the lump whose molecular mass is largest was isolated from the reaction system with ultrafiltration membrane to simplify the four lumps system to three lumps sub-reaction system.

Experimental conditions for model verification: The initial substrate at 35 °C was the mixture of BSA and the hydrolysates, and the mass concentration was 4.6 mg mL^{-1}. The hydrolysis was preceded at pH 8.0 ($\zeta_{enz./sub.}$ = 1/80). The initial substrate at 45 °C was similar to that at 35 °C, and the mass concentrate was 4.9 mg mL^{-1} ($\zeta_{enz./sub.}$ = 1/120). The sampling time is different from that in the experiment for parameter estimation.

Parameter estimation

There are six kinetic parameters to be calculated in the four lumps network of tryptic hydrolysis of BSA. But the number of equations to describe the network is four, which is less than the number of the parameters. Therefore, estimating all the pa-

rameters simultaneously will result in the problem of multivalue dependency. In order to avoid the problem, the kinetic constants of the model were estimated by applying a partial nonlinear least squares estimator in two estimation steps. First of all, the parameters of the sub-reaction system, which contains lumps B, C and D, were calculated. The number of parameters is reduced to three (e.g. k4–k6). And the number of equations to describe the sub-reaction network is three, too. It is not difficult to calculate the three rate constants. Then the residual parameters (e.g. k1–k3) can be estimated easily because k4–k6 has been fixed. Table 2 gives the results of parameter estimation.

Table 3 shows that there are several products for the lump whose molecular mass are large. The rate of generating different small lumps from the large lump is different. The rate of yielding the lump, whose molecular mass is large, is faster than that of the lump whose molecular mass is small. For example, the rate constant of yielding lump B from lump A is larger than that of yielding lump C. The reason is that hydrolysis reaction rate is affected by the decomposition of the enzyme-substrate complex. The combination position of substrate and enzyme is not random, and inclined to form the complex that can decompose easily. It is easy to be set free from the complex for the product whose volume is large because of the space repulsion. As a result, the apparent rate is fast for this kind of reaction.

Table 3 – *Results of model parameter estimation*[a]

Reaction	Rate coefficient	30 °C	40 °C	50 °C
A → B	k_1	0.1404	0.3266	0.6464
A → C	k_2	0.02148	0.03383	0.04987
A → D	k_3	0.01264	0.02439	0.04018
B → C	k_4	0.03201	0.06041	0.1332
B → D	k_5	0.01306	0.02939	0.05086
C → D	k_6	0.02685	0.05738	0.0963

[a]The unit of k is mL mg^{-1} min^{-1}

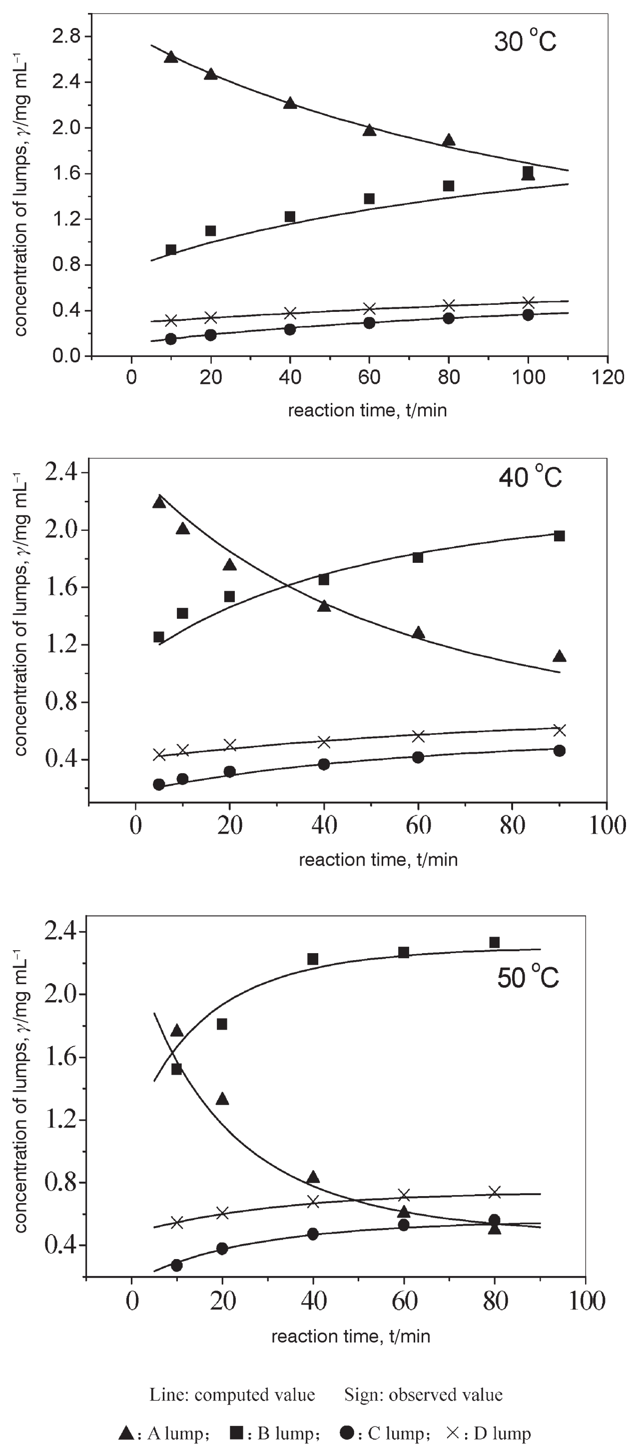
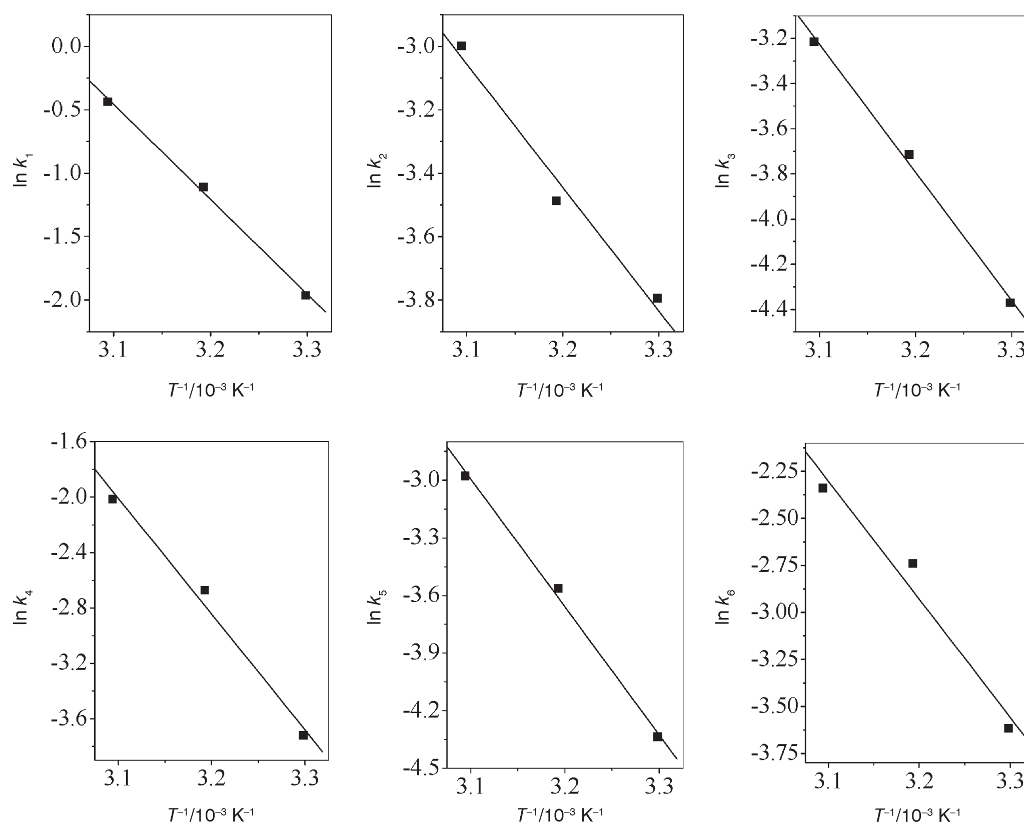


Fig. 3 – *Comparison between observed and computed values at different temperatures*

Figure 3 is the comparison of the observed values of each lump with the computed values for parameter estimation at 30 °C, 40 °C and 50 °C, respectively. The initial reaction rates rise along with the temperature's rising. But the reaction rates decrease faster at high temperature than at low temperature. It is due to the faster deactivation rate of trypsin at high temperature. In addition, it can be concluded from figure 3 that the decreasing amount of lump A equals to the increasing summation of lump B, C and D. The concentration of lump B and C goes up with time since the decomposing rates of them are lower than the generating rates. Compared with lump C and D, the generating rate of lump B is fast, which also shows that the hydrolysates with large volume can be produced easily.



F i g . 4 – *Relationship between ln k and temperatures*

The increasing extent of reaction rate for each lump is less than twice when the temperature rises 10 °C. The different extent for each lump means the influence of temperature on reaction rate is different because the reactive capacity of each lump is affected by its structure. When the reaction conditions of substrate concentration, ratio of enzyme to substrate and pH are settled, the rate constant is the function of temperature, and follows Arrhenius equation. The relationship between rate constants of each lump and reaction temperatures is shown in figure 4. The straight-line in figure 4 indicates that the relationship can fit the Arrhenius equation well. The reaction activation energy for each lump can be calculated by Arrhenius equation, and that of the lumps is between 45 kJ mol^{-1} to 65 kJ mol^{-1}.

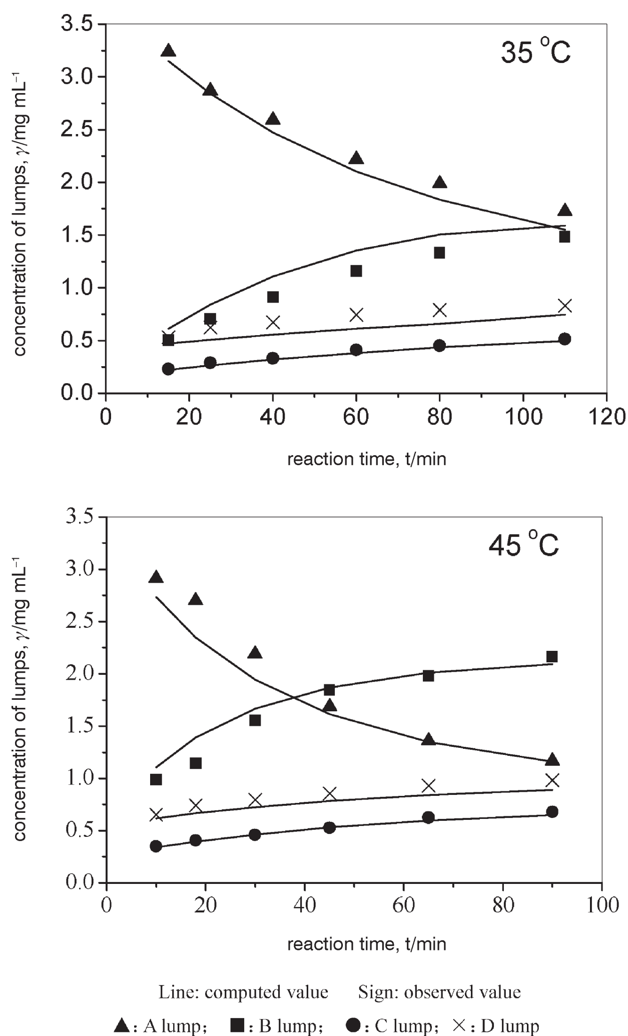
Verification of the lumping model

In order to examine the ability of prediction and extrapolation of the kinetic model and the good quality of the obtained rate constants, hydrolysis experiment was preceded at other conditions different from that of estimating the parameters. For example, the composition of initial substrate, reaction temperatures, and sampling time are changed. The results of comparing observed values with computed values show, that the kinetic model can predict the mass concentration of each lump effectively. Figure 5 is the results of the comparison of experimental values and computed values at 35 °C and 45 °C, respectively.

There are some deviation between observed values and computed values from the kinetic model. In the simplification process of the intrinsic kinetic equation, it is assumed that the value of the denominator of Eq. 4 does not change with time and substrate inhibition and product inhibition are ignored, which is one of the reasons that introduce the errors to the kinetic model. In addition, the quantitative analysis of mass concentration for the hydrolysates is not precise enough owing to the multi-component complexity of products. Furthermore, the rate constants at 35 °C and 45 °C, calculated by Arrhenius equation, are not very accurate.

Conclusion

In order to investigate the kinetic behavior of the components in a complicated system, a numerical rule was adopted to reduce the dimensionality of kinetic model by lumping species. A simplified kinetic model for bovine serum albumin tryptic hydrolysis was established on the basis of the mechanism of protein enzymatic hydrolysis. Some hypothesis about the mechanism of the reaction and



F i g . 5 – *Comparison between observed values and computed values at different temperatures*

the lumping criteria were made before the building up of the model. The reaction of the lumps was described with simplified kinetic equation, which ignores the influence of product inhibition and substrate inhibition. The rate coefficient were calculated by applying a partial nonlinear least squares estimator in two estimation steps. The adequacy of the kinetic model was tested by comparing the computed values with the experimental values. The results showed that the kinetic model could predict the distribution of the mass concentration of products well. The establishment of the simplified kinetic model will help to optimize the operational conditions to yield the target product and will supply reference to the kinetic study of complicated biochemical reaction. But the range of use for this kind of kinetic model is limited because it is not an intrinsic kinetic model.

Notation

γ_{E0} – initial mass concentration of enzyme, mg mL^{-1}
E – free enzyme
EP – inert complex
ES – active middle complex
ES_2 – inactive complex
k_{cat} – catalytic coefficient
k_d – deactivation coefficient of enzyme
K_m – Michaelis constant,
Kp – product inhibition constant
Ks – substrate inhibition constant
P – product of protein enzymatic hydrolysis
S – substrate
ζ – mass ratio, m_{enz}/m_{sub}

References

1. *Akemichi Ueno, Yeong-Man Hong, Naokatu Arakaki,* J. Biochem. **98** (1985) 269.
2. *Masakazu Takahashi, Shigeo Moriguchi, Toshiko Minami,* Lett. Peptide Sci. **5** (1998) 29.
3. *Carraway, R. E., Mitra, S. P., Cochrane, D. E.,* J. Biol. Chem. **262** (1987) 5968.
4. *Cao, G., Viola, A., Baratti, R.,* Appl. Catal. **41** (1988) 301.
5. *Weng Huixin, Ouyang Fusheng, Ma Jun,* J. Chem. Ind. & Eng. (China). **46** (1995) 663.
6. *Froment, G. F.,* Catal. Today. **52** (1999) 153.
7. *James C. L., Edwin N. L.,* Biotech. Bioeng. **31** (1988) 869.
8. *He Zhimin, Zhang Ziding, He Mingxia,* Process. Biochem. **10** (2000) 1235